Method f r Staining Biological Specimens by Combining Unstable Reagents on a Microscope Slide

Related Applications

This application is a continuation in part of U.S. Application Serial No. 60/087,673 filed June 2, 1998.

Field of the Invention

The present invention relates to formulations for component histochemical staining solutions as well as methods for formulating, storing, and combining components of unstable histochemical staining solutions. In particular, the present invention relates to methods for combining component histochemical solutions directly on the biological sample of interest.

Background

Histochemistry is the science by which chemical reactions are used to identify particular substances within a cell. One method of identifying particular substances in the cell is by staining the cells with chemicals (dyes) that make such substances or structures more visible. Perhaps the most common stain materials are hematoxylin and eosin. Hematoxylin is utilized to stain the nuclei of the cell dark blue while eosin stains the cell cytoplasm various shades of red or yellow that contrast with the blue stain of the nuclei. Other stains can be used to identify other substances within the cell such as collagen, elastin, mucin, ferric iron, and other substances. Still other stains can be used to identify agents that infect the human

body such as bacteria and fungi. Many of the stains used to identify certain substances and/or structures within or outside the cell require the used of stains that are unstable, toxic, and generally messy and difficult to work with.

Presently, many automated and manual histochemical staining protocols require the pre-mixing of two or more solutions prior to staining sample tissue. In many cases the mixing of several solutions to prepare a single solution for staining produces a staining solution that is inherently unstable. Instability may manifest itself by the appearance of precipitates or films in the staining solution. For example, many silver staining solution are photolabile. Ammoniacal silver solutions degrade rapidly and a silver residue can be observed on top of the solution within hours of mixing. The formation of films and precipitates negatively affects the staining of the tissue and therefore decrease the accuracy of histochemical testing. Furthermore, the daily preparation of fresh histochemical staining solutions is time consuming. It may also be costly since expensive reagents such as silver nitrate may be squandered if staining solution is prepared and not used by the end of the day. Therefore, there exists a need for improved histochemical staining methods that employ unstable staining solutions.

The present invention obviates the need to prepare new staining preparations on a daily basis. The present invention permits the mixing of component histochemical staining solutions on a sample tissue slide, solutions that have previously been combined in the laboratory prior to staining a slide sample. Unlike the combined solutions which are unstable, the separated component solutions are stable for long periods. The component histochemical solutions of the present invention may be stored as separate solutions for long duration and may then be

combined on sample tissue that has been placed on a microscope slide. The results of tissue assays using the component histochemical staining solutions of the present invention are equal to or better than manual or automated methods utilizing completely mixed standard histochemical staining solutions.

Summary of the Invention

The present invention relates to automated methods for staining biological materials on a slide comprising the use of component histochemical solutions combined directly on a biological sample of interest. In one embodiment, the method comprises providing at least two stable solutions that together comprise an unstable staining solution, sequentially delivering the stable solutions to a biological sample of interest on a planar surface, and combining the stable solutions directly on the biological material of interest to effectuate staining of the material. In specific embodiments, methods are provided for automated silver staining, iron staining, trichrome staining, and mucicarmine staining.

In various preferred embodiments, the biological material is selected from the group consisting of tissue sections, tissue culture cells, nucleic acids, proteins, and chromosomes; the unstable staining solution is selected from the group consisting of fungi staining solutions, silver staining solutions, trichrome staining solutions, mucin stains, mucicarmine staining solutions, iron staining solutions, Verhoff's staining solution, and Steiner staining solution, the solutions are mixed, and the mixing is accomplished by applying a gas stream to the at least first and second stable solutions on the biological material.

Detailed Description of the Preferred Embodiments

The present invention relates to automated methods for staining biological materials on a surface comprising the use of component histochemical solutions mixed directly on a biological sample of interest. There are many histochemical staining procedures that require the use of a staining solution that is made of several component solutions. These component solutions are mixed together prior to being placed on a microscope slide containing a tissue section of interest. In the present invention these component solutions are kept in separate containers and only mixed after placement of each solution successively on the biological material of interest. In a preferred embodiment, the solutions are mixed on the slide by an automated histochemical instrument and the concentrations of the solutions optimized for the instrument and the method of mixing. The methods of the present invention do not require mixing of the solutions, but such mixing speeds up and limits variation in the resulting solution.

As used herein, the term "solution" encompasses solutions, emulsions, and suspensions.

As used herein, the term "stable" means that the solution can be stored and re-used, and thus does not need to be made fresh prior to use. Preferably, a "stable solution" has a shelf-life of at least one week.

As used herein, the term "unstable" means that the solution exhibits diminished capacity to stain the target organism or tissue, upon standing for any period of time, even as little as one hour. For example, many silver staining

solutions are photolabile and heat labile. Similarly, many staining solutions change color or form precipitates or films as a result of oxidation, such as iron hematoxylin, and must be discarded after use. The methods of the invention apply to any unstable multi-component staining solution that can be made by mixing two or more stable sub-components together. Special stains color, or coat with metals, certain specific kinds of cells or cellular structures. This is done by applying, in sequence, dyes and other chemicals (oxidizers, reducing agents, metals) until the targeted staining is accomplished. Some stains employ as many as 10 different solutions. Each solution is termed a component of the stain.

Some individual components of the stain are made of "sub-components". If a final formulation of a solution cannot be stored until it is needed for use, then the separate ingredients must be made into "stock solutions" and combined immediately before use. The combined solution is not "stable", so it must be used within a short time, before it degrades and does not perform its function in the staining procedure. This "unstable" combined solution is called a "working solution". A single component of a stain may have multiple sub-components that can be combined in a variety of ways to achieve the desired result.

The method of the present invention can be used with any histochemical solution that exhibits diminished capacity to stain the target organism or tissue, upon standing for any period of time, even as little as one hour. Such unstable multi-component staining solutions include, but are not limited to fungi staining solutions, silver staining solutions, iron staining solutions, iron hematoxylin solutions, trichrome staining solutions, mucin stains, mucicarmine staining solutions, Verhoff's staining solution, amyloid staining solutions, and Steiner staining solution.

See for example, Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (McGraw-Hill, New York, Lee G. Luna, Ed. (1968)) (AFIP Manual); Theory and Practice of Histological Techniques (Churchill Livingston, NY, ed. by Bancroft and Stevens, 4th edition, 1996); both incorporated by reference herein in their entirety. The method of the present invention can be used for bi-component, tri-component (as in the example above) and other multi-component unstable histochemical solutions.

For example, the conventional Grocott's method for fungi (GMS) requires a number of solutions to accomplish the staining of fungal tissue (chromic acid, sodium bisulfite, gold chloride, sodium thiosulfate, light green solution). In addition, this staining protocol requires the use of a methanamine-silver nitrate-borax solution which is made by mixing a silver nitrate-methanamine stock solution (0.25% silver nitrate, 2.85% methanamine) with a 5% borax solution to produce a working methanamine-silver nitrate solution (0.125% silver nitrate, 1.425% methanamine, 0.2% borax). While the stock methanamine-silver nitrate solution is stable, the working solution is unstable and thus must be made fresh every day.

In the present invention, the silver nitrate solution is kept separate from the methenamine-borax solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the silver nitrate solution is comprised of from about 0.2% to about 1.0% silver nitrate. In a preferred embodiment of the present invention the methenamine-borax solution is from about 2.0% to about 4.0% methenamine and from about 0.2% to about 0.6% borax in distilled water. In a preferred embodiment of the present invention the silver nitrate solution is added to the sample and after addition of a liquid coverslip solution, an

equal volume of methenamine-borax solution is added to the sample. Also, each of the three sub-components can be added as separate solutions to the sample. Furthermore, one of skill in the art will recognize that a methenamine-silver nitrate stock can be mixed on the tissue with the borax solution.

Similarly, conventional ammoniacal silver staining requires the use of both silver nitrate and ammonium hydroxide/sodium hydroxide solutions. While the stock silver nitrate and ammonium hydroxide/sodium hydroxide solutions are stable, the combined working solution is unstable and thus must be made fresh every day.

In the present invention, the silver nitrate solution is kept separate from the ammonium hydroxide/sodium hydroxide solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the silver nitrate solution is comprised of from about 0.2 % to about 1.0 % silver nitrate. In a preferred embodiment of the present invention the ammonium hydroxide/sodium hydroxide solution is from about 0.3 % to about 1 % ammonium hydroxide and from about 0.1% to about 0.5% sodium hydroxide in distilled water. Also, each of the three sub-components can be added as separate solutions to the sample.

Trichrome staining and mucicarmine staining require both Weigerts iron hematoxylin A and B solutions. While the stock Weigerts A and B solutions are stable, the combined working solution is unstable and thus must be made fresh every day.

In the present invention, the Weigerts A solution is kept separate from the Weigerts B solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the Weigerts A solution

is comprised of from about 0.7 % to about 1.5 % hematoxylin in 95% alcohol. In a preferred embodiment of the present invention the Weigerts B solution is from about 0.7% to about 1.5 % aqueous ferric chloride and from about 0.5 % to 1.5 % HCl in distilled water. In a preferred embodiment of the present invention the Weigerts B solution is added to the sample and after addition of a liquid coverslip solution, an equal volume of Weigerts A solution is added to the sample.

Gomori's iron staining require both potassium ferrocyanate and hydrochloric acid solutions. While the stock solutions are stable, the combined working solution is unstable and thus must be made fresh every day.

In the present invention, the potassium ferrocyanate is kept separate from the hydrochloric acid solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the potassium ferrocyanate solution is comprised of from about 8 % to about 12 % potassium ferrocyanate in 95% distilled water. In a preferred embodiment of the present invention the hydrochloric acid solution is from about 15 % to about 30 % HCl in distilled water.

In the methods of the invention, the solutions can be contacted with the biological material for widely varying periods of timing to accomplish the object of staining the specimen. In one embodiment, the solution is contacted with the biological specimen for between about one second and about one hour, preferably for between about 10 seconds and 45 minutes, and most preferably for between about one minute and 30 minutes.

The methods of the present invention can be performed over a wide temperature range. In one embodiment, the methods can be performed at between

about 20°C to about 90°C; more preferably at between about 40°C to about 70°C; and most preferably between about 50°C and about 60°C.

The parameters of temperature at which the staining is carried out, and the duration of contacting the biological specimen with the solution, can be varied extensively depending upon the stain, the biological specimen, and the instrumentation used, as will be appreciated by one of skill in the art.

In a preferred embodiment of the present invention the solutions are added to the sample tissue by an automated mechanism which can mix the solutions on the slide. Such automated instruments include those described in U.S. Patent Nos. 5,595,707; 5,654,199; 5,654,200 and 5,650,327 herein incorporated by reference in their entirety. The particular concentrations of reagents in the component solutions can be optimized by standard experimental design to provide optimum ranges of concentrations, oxidation/reduction potentials, ionization, and/or pH.

In a preferred embodiment, the methods of the present invention are automated. Manual and most robotic staining is performed by dipping the slides into open vessels that are filled with pre-mixed solutions of dyes and chemicals. A variant of this technique is flooding chambers containing the slides with the pre-mixed solutions. In contrast, in the method of the present invention, the slide or other surface is itself used as the container for the staining solution. The slides are positioned flat, biological material side up, and aliquots of staining solutions are sequentially delivered and mixed on the biological material. Instrumentation for conducting such automated staining includes, but is not limited to the NexEs™ system (Ventana Medical Systems, Tuscon, AZ) and that disclosed in U.S. Patent

Nos. 5,654,200, 5,650,327, 5,654,199, and 5,595,707, all hereby incorporated by reference in their entirety.

In a preferred embodiment, methods are used to apply a layer over the "pool" of staining solution to prevent evaporation, regulate temperature, and enhance mixing, such as that described in U.S. Patent Nos. 5,654,200, 5,650,327, 5,654,199, and 5,595,707, all hereby incorporated by reference in their entirety. In a particularly preferred embodiment, the layering method comprises (a) covering the sample with an aqueous surface layer by applying an aqueous solution to a planar surface adjacent a biological sample mounted thereon; and (b) covering the aqueous surface layer with an evaporation inhibiting liquid layer by applying the evaporation inhibiting liquid to the planar support surface adjacent the biological sample in an amount sufficient to form a continuous layer of evaporation inhibiting liquid over the sample. The evaporation inhibiting liquid is substantially water-insoluble, substantially water-immiscible and substantially non-viscous; has a specific gravity less than water, and a boiling point above 50o C.; and is devoid of chemical characteristics which would significantly interfere with biochemical reactions carried out on the sample. The biological sample can then be optionally treated (c) with an aqueous reagent solution by applying the reagent solution to the planar support surface adjacent the biological sample. The reagent solution flows to the biological sample under the evaporation inhibiting liquid layer, and the sample is continuously protected from dehydration by the evaporation inhibiting layer.

The methods of the present invention include mixing the stable solutions on the surface of the biological sample. In a preferred embodiment, this is accomplished by applying at least one gas stream to an area of the surface of the

evaporation inhibiting liquid layer between the center of the evaporation inhibiting layer and the edge of the planar support surface, the gas stream having a central axis forming an acute angle with the planar support surface. According to one embodiment of the present invention, the reagent solution is preferably stirred by a vortex formed by applying two off-center gas streams, flowing in opposite directions, to the surface of the evaporation inhibiting liquid layer. According to a further embodiment of the present invention, the stable solutions are stirred by a vortex formed by applying a single gas stream along a longitudinal edge of the slide, the gas stream originating from the distal edge of the slide.

Biological materials that can be stained by the methods of the invention include, but are not limited to tissue sections, tissue culture cells, cell components, including cell organelles, chromosomes, nucleic acids, carbohydrates, lipids, and proteins, smears of blood, sputum, and other body fluids, excretions and secretions, and micro-organisms including parasites, viruses, bacteria, and fungi.

The methods of the present invention can also utilize newly developed stains. A generic method for applying the subject invention to most any stain comprises:

1. Reviewing the staining literature and selecting a particular staining protocol.
2. Evaluate the instrument platform to be used to determine parameters and limitations in time, temperature and rinsing and mixing events available on the instrument.
3. Adapt the staining procedure to conform to the instrument parameters. Example: If more or less time is needed, but not available, then increase or decrease, temperature or reagent concentration.
4. Test the modified staining procedure and evaluate the result.
5. If the result is sub-optimal, then identify the component responsible for the problem.
6. Substitute or reformulate the reagent to compensate for the problem.
7. Retest and reevaluate in a loop until stain is optimized.

This procedure will permit one of skill in the art to apply the subject invention to most any staining procedure, including those described in Theory and Practice of Histotechnology, Dezna C Sheehan H.T (ASCP), Battelle Press, 2nd ed., 1980; and Laboratory Histopathology, Anthony E. Woods & Roy C. Ellis, Churchill Livingstone, 1st ed., 1994; both incorporated by reference herein in their entirety.

The following Examples are presented for illustrative purposes only and are not intended, nor should they be construed as limiting the invention in any way. Those skilled in the art will recognize that variations on the following can be made without exceeding the spirit or scope of the invention.

Example 1

Grocott's Method for Fungi (GMS) Staining

A solution of 0.35% silver nitrate was made by adding 0.35g silver nitrate to 100 ml de-ionized water. A solution of 3.0% methenamine and 0.4% borax was made by dissolving 3g methenamine and 0.4g of borax in 100 ml of distilled water. A solution containing 0.5% sodium bisulfite was made by dissolving 0.5g of sodium bisulfite in 100 ml of distilled water. A solution containing 3.75% chromic acid was made by dissolving 3.75g of chromium trioxide in 100 ml of distilled water. A solution containing 0.2% gold chloride was made by dissolving 0.2g of gold chloride in 100 ml of distilled water. A solution containing 2.0% sodium thiosulfate was made by dissolving 2.0g of sodium thiosulfate in 100 ml of distilled water. A 0.05% light green solution was made by diluting 25 ml of stock solution (2g of light green dissolved in 99 ml distilled water and 1 ml glacial acetic acid) in 100 ml of de-ionized water. 200 µl of the silver nitrate solution and 200 µl of the methenamine-borax solution were dispensed onto a tissue sample mounted on a microscope slide

using an automated histochemical dispensing apparatus (Ventana Medical Systems, Inc., Tucson Arizona). The sample tissue, *Aspergillus ctyptococcus* was prepared for staining by standard technique. The sample tissue was place in the automated histochemical staining instrument and the following protocol was used in staining the tissue.

In this example, as well as the four succeeding examples, the wash solution was comprised of 0.2% Tween 20 in de-ionized water (pH to 7.0 ± 0.5). Liquid coverslip is 99.99% Norpar 15 and less than 0.01% oil red 0.

TABLE 1

1. Warm-up rinse buffer to 41.0° C
2. Rinse slide
3. Adjust slide volume
4. Apply liquid coverslip
5. Warm slide chamber to 60.0° C
6. Rinse slide
7. Adjust slide volume
8. Apply liquid coverslip
9. Start timed steps
10. Rinse slide
11. Adjust slide volume
12. Apply 200µl of 4% chromic acid and incubate for 15 minutes
13. Apply liquid coverslip
14. Rinse slide
15. Adjust slide volume
16. Apply 200µl of 0.5% sodium bisulfite and incubate for 3 minutes
17. Apply liquid coverslip
18. Rinse slide
19. Rinse slide
20. Adjust slide volume
21. Apply 200 µl of 0.5% silver nitrate solution and incubate for 3 minutes
22. Apply liquid coverslip
23. Apply 200 µl of 4% methanamine/0.4% borax solution and incubate for 18 minutes
24. Apply liquid coverslip
25. Rinse slide
26. Adjust slide volume

27. Apply liquid coverslip
28. Apply 200µl of 0.2% gold chloride and incubate for 3 minutes
29. Apply liquid coverslip
30. Rinse slide
31. Adjust slide volume
32. Apply 200µl of 2.5% sodium thiosulfate solution and incubate for 3 minutes
33. Apply liquid coverslip
34. Rinse slide
35. Adjust slide volume
36. Apply liquid coverslip
37. Apply 200µl of light green solution and incubate for 3 minutes
38. Apply liquid coverslip
39. Rinse slide

A visual comparison between the tissue prepared using the automated protocol as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for GMS described in the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (McGraw-Hill, New York, Lee G. Luna, Ed. (1968)) (AFIP Manual). Such comparison showed that the tissue stained with the component solutions on the automated system were cleaner, *i.e.* no black ring around the sample tissue. All tissue samples remained attached to the slide, whereas those stained manually started to lift off the slide. The staining contrast was better on the slides stained with the component solutions.

The silver nitrate solution and methanamine/borax solutions were stored at 4° C for three months, after which time the protocol in table 1 was re-run on similar tissue. A visual comparison was made between the tissue prepared as described above using the stored solutions and the manual staining of identical tissue with freshly made solutions. The staining comparison demonstrated that the tissue

stained by the stored solutions run on the automated system were comparable or better than tissue stained manually with freshly made solutions.

Example 2

Ammoniacal Silver Staining

A 10% silver nitrate stock solution was made by dissolving 10g of silver nitrate in de-ionized water. A working solution of 0.2% silver nitrate was made by diluting 2 ml of 10% stock solution with 48 ml of de-ionized water. An ammonium hydroxide/sodium hydroxide solution was made by dissolving 9.20 ml of 1N ammonium hydroxide and 3.60 ml of a 3% sodium hydroxide in 37.2 ml of de-ionized water. A solution containing 0.5% potassium permanganate was made by dissolving 0.5g of potassium permanganate in 100ml of distilled water. A solution containing 0.5% oxalic acid was made by dissolving 0.5g of oxalic acid in 100ml of distilled water. A solution containing 2.5% ferric ammonium sulfate was made by dissolving 2.5g of ferric ammonium sulfate in 100ml of distilled water. A solution containing 10% formalin was made by diluting 10 ml of concentrated formaldehyde (37-40%) in 90 ml distilled water . A solution of 0.2% gold chloride was made by dissolving 0.2g of gold chloride in 100ml of distilled water. A solution containing 2.0% sodium thiosulfate was made by dissolving 2g of sodium thiosulfate in 100 ml of distilled water. A solution of 1.5g/L nuclear fast red was made by dissolving 0.15g of nuclear fast red in 5% solution of aluminum sulfate (5g of aluminum sulfate in 100 ml distilled water) over heat. 200µl of 0.2% silver nitrate and 200µl of the ammonium hydroxide/sodium hydroxide solution were dispensed onto a tissue sample mounted on a microscope slide using an automated histochemical dispensing

apparatus (Ventana Medical Systems, Inc., Tucson Arizona). Sample liver tissue was prepared according to standard protocol. The sample tissue was placed in the automated histochemical staining instrument and the following protocol was used to stain the tissue.

TABLE 2

1. Warm-up rinse buffer to 41.0° C
2. Rinse slide
3. Adjust slide volume
4. Apply liquid coverslip
5. Warm slide chamber to 60.0° C
6. Rinse slide
7. Adjust slide volume
8. Apply liquid coverslip
9. Rinse slide
10. Adjust slide volume
11. Apply 200µl of 0.5% potassium permanganate and incubate for 3 minutes
12. Apply liquid coverslip
13. Rinse slide
14. Adjust slide volume
15. Apply 200µl of 0.15% oxalic acid and incubate for 3 minutes
16. Apply liquid coverslip
17. Rinse slide
18. Adjust slide volume
19. Apply 200µl of 2.5% ferric ammonium sulfate solution and incubate for 3 minutes
20. Apply liquid coverslip
21. Rinse slide
22. Apply 200µl of 0.2% silver nitrate solution and incubate for 3 minutes
23. Apply 200µl of ammonium hydroxide/sodium hydroxide solution and incubate for 3 minutes
24. Apply liquid coverslip
25. Rinse slide
26. Rinse slide
27. Adjust slide volume
28. Apply 200µl of 10% formalin solution and incubate for 3 minutes
29. Apply liquid coverslip
30. Rinse slide
31. Adjust slide volume
32. Apply 200µl of 0.2% gold chloride and incubate for 3 minutes
33. Apply liquid coverslip

34. Rinse slide
35. Adjust slide volume
36. Apply 200µl of 2.0% sodium thiosulfate and incubate for 3 minutes
37. Apply liquid coverslip
38. Rinse slide
39. Adjust slide volume
40. Apply 200µl of 1.5 g/L nuclear fast red and incubate for 3 minutes
41. Apply liquid coverslip
42. Rinse slide

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for reticulum stain described in the AFIP Manual. Tissue stained by both techniques appeared to be identical.

The silver nitrate solution and ammonium hydroxide/sodium hydroxide solutions were stored at 4° C for two months, after which time the protocol in table 1 was re-run on similar tissue. A visual comparison between the tissue prepared as described above using the stored solutions was compared with manual staining of identical tissue with freshly made solutions carried out in accordance with the protocol for reticulum stain described in the AFIP Manual. The staining comparison demonstrated that the tissue stained by stored solutions run on the automated system were comparable or better than tissue stained manually with freshly made solutions.

A visual comparison between the tissue prepared as described above using the stored solutions was compared with manual staining of identical tissue with one day old solutions carried out in accordance with the protocol for reticulum stain described in the AFIP manual. The staining comparison demonstrated that the tissue stained by stored solutions run on the automated system was significantly better than tissue stained manually with the one day old solutions.

Example 3

Masson's Trichrome Stain

Solutions for Masson's Trichrome stain were made as follows. Weigert's iron hematoxylin solution A was made by adding 1g of hematoxylin to 100 ml of 95% alcohol. Weigert's iron hematoxylin solution B was made by adding 4 ml of 29% aqueous ferric chloride, 95 ml of distilled water and 1 ml hydrochloric acid together. Biebrich's scarlet-acid fuchsin solution was made by combining 90 ml of 5% aqueous solution of Biebrich's scarlet with 10 ml of 10% aqueous acid fuchsin and 1 ml glacial acid. The resultant solution was mixed and filtered through a Whatman 3 filter paper.

The 1% phosphotungstic acid solution was made by combining 1g phosphotungstic acid in 100 ml of de-ionized water. The aniline blue solution was made by adding 0.4g aniline blue to 100 ml of distilled water and 1ml of acetic acid. The acetic acid solution was made by adding 0.5ml acetic acid to 100 ml of distilled water.

Table 3

1. Warm-up rinse buffer to 41.0° C
2. Rinse slide
3. Warm slide chamber to 60.0° C
4. Rinse slide
5. Apply 300 µl of Weigerts B solution (1x) and incubate for 3 minutes
6. Apply liquid coverslip
7. Apply 200 µl of Weigerts A solution (1x) and incubate for 6 minutes
8. Apply liquid coverslip
9. Rinse slide
10. Rinse slide
11. Apply 200 µl of 5% Biebrich Scarlet solution and incubate for 9 minutes

12. Apply liquid coverslip
13. Rinse slide
14. Apply 300µl of 1% phosphotungstic acid solution and incubate for 6 minutes
15. Apply liquid coverslip
16. Rinse slide
17. Apply 200µl of 0.40% aniline blue and incubate for 3 minutes
18. Apply liquid coverslip
19. Rinse slide
20. Apply 300 µl of 0.5% acetic acid and incubate for 3 minutes

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for Masson's trichrome stain described in the AFIP Manual. The staining comparison demonstrated that the tissue stained by stored solutions when run on the automated system was comparable to tissue stained manually with freshly made solutions.

Example 4

Mucicarmine Staining

Solutions for mucicarmine staining were made as follows. Mayer's stock mucincarmine solution was made by combining 1g carmine and 0.5g anhydrous aluminum chloride in a Pyrex beaker and adding 2 ml distilled water. The solution was heated over a small flame and agitated with a glass rod for approximately 2 minutes until the solution turned purple and had the consistency of syrup. Thereafter, 100 ml of 50% ethanol was added to the syrupy mixture and the solution was incubated at room temperature for 24 h. The solution was filtered through Whatman 3 filter paper.

TABLE 4

1. Warm-up rinse buffer to 41.0° C
2. Rinse slide
3. Adjust slide volume
4. Apply liquid coverslip
5. Warm slide chamber to 60.0° C
6. Rinse slide
7. Adjust slide volume
8. Apply liquid coverslip
9. Start timed steps
10. Rinse slide
11. Adjust slide volume
12. Apply 300 µl of Weigerts B solution and incubate for 3 minutes
13. Apply 200 µl of Weigerts A solution and incubate for 3 minutes
14. Apply liquid coverslip
15. Rinse slide
16. Adjust slide volume
17. Apply 200 µl of mucicarmine solution and incubate for 6 minutes
18. Apply liquid coverslip
19. Rinse slide
20. Adjust slide volume
21. Apply liquid coverslip
22. Apply 100 µl of 0.1% tartrazine solution and incubate for 3 minutes
23. Apply liquid coverslip
24. Rinse slide

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for mucicarmine stain described in the AFIP Manual. Tissue stained by both techniques appeared to be identical.

Example 5

Gomori's Iron Stain

A solution of 1.5g/L nuclear fast red was made by dissolving 0.15g of nuclear fast red in 5% solution of aluminum sulfate over heat. A solution of 20% hydrochloric acid was made by adding 20 ml concentrated hydrochloric acid with 80 ml distilled water. A 10% solution of potassium ferrocyanide was made by dissolving 10g potassium ferrocyanide in 100 ml distilled water.

TABLE 5

1. Warm-up rinse buffer to 41.0° C
2. Rinse slide
3. Adjust slide volume
4. Apply liquid coverslip
5. Warm slide chamber to 60.0° C
6. Rinse slide
7. Adjust slide volume
8. Apply liquid coverslip
9. Start timed steps
10. Rinse slide
11. Adjust slide volume
12. Apply 200 µl 10% potassium ferrocyanate
13. Apply 200 µl of 20.0% hydrochloric acid and incubate for 9 minutes
14. Apply liquid coverslip
15. Rinse slide
16. Adjust slide volume
17. Apply 100 µl of 1.5% Nuclear fast red solution and incubate for 3 minutes
18. Apply liquid coverslip
19. Rinse slide

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for Gomori's iron stain described in the AFIP Manual. Tissue stained by both techniques appeared to be identical.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.